Antivascular antibody

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The sera from many patients with various connective tissue diseases contain distinctive antibodies. The demonstration of some, particularly antinuclear antibody (ANA) and rheumatoid factor (RF) have proved to be practical and extremely useful in helping to categorize patients as to disease state and in following the clinical progress of patients with connective tissue diseases.

Human sera reactive with the small vessels in mouse liver sections were first observed in this laboratory in 1965 when the screening of sera for ANA was established as a routine procedure. Of the total number of specimens obtained from a number of different clinic and hospital patient populations, approximately 5,295 have been tested for ANA. The reactivity observed with mouse capillaries was initially termed 'vessel' or 'vascular staining' to distinguish it from various other types of immunofluorescence observed in liver sections.

This phenomenon has been shown to be due to an IgG antibody to vascular tissue, and patients whose sera contain this activity have been reviewed to assess the clinical significance of antivascular antibody.

Materials and methods

Antinuclear antibodies (ANA) were detected by reacting sera with mouse liver sections and fluorescein conjugated antihuman IgG. Antisera to human plasma Cohn Fraction II (Squibb & Sons, N.Y.) were produced in rabbits using multiple intradermal injections of Fraction II in complete Freund's adjuvant followed by multiple intravenous injections of Fraction II in 0.15 mol/l. NaCl for 2 to 3 weeks. Antisera were collected after a 5- to 10-day rest period and after multiple courses of intravenous injections of Fraction II. Reactivity of the antiserum was shown to be specific for IgG in gel diffusion and immunoelectrophoresis with IgG, IgM, and whole human serum. The antisera were conjugated with fluorescein isothiocyanate (FITC, Isomer 1, Sigma Chemical Co., St. Louis, Mo.), 25 µg FITC/1 mg protein. Unconjugated FITC was removed by dialysis and filtration on Sephadex G-25 (Pharmacia Fine Chemicals, Inc., Piscataway, N.J.) and the resultant labelled antisera were adsorbed three or more times with mouse liver

powder (75 µg/ml) to remove nonspecific staining. Cryostat sections of mouse liver 4 µm thick were exposed to patients' sera for 30 minutes at room temperature and then washed three times with 0.005 mol/l. phosphate buffer, pH 7.1, containing 0.15 mol/l. NaCl (PBS). The sections were then treated with rabbit antihuman Cohn Fraction II-FITC for 30 minutes and washed three times with PBS. Routine testing was done at a 1:4 dilution of the patients' sera in PBS and all sections were viewed in a Leitz microscope equipped for fluorescence microscopy.

Selected sera which reacted with mouse vessels were also tested on sections of mouse kidney and stomach. Several sections were first reacted with sera containing AVA and then with fresh normal human serum and counter stained with goat antihuman C3-FITC (Hyland Laboratories, Los Angeles, Calif.). Unconjugated rabbit antihuman globulin was used in several blocking experiments. Three positive serum specimens were injected intravenously into mice and sections of their kidneys were examined for basement membrane staining by anti-IgG-FITC. Five positive sera were reacted with sections of three different human kidneys obtained at operation. Five positive specimens were layered on sucrose gradients (10-40%, w/v) and centrifuged at 147,000 × g (max) for 18 hours in a SW 50.1 rotor (Spinco Div., Beckman Instruments, Inc., Palo Alto, Calif.). The gradients were fractionated by bottom puncture and 0.8 ml fractions collected for assay of vascular reactivity and for quantitation of IgG and IgM by radial immunodiffusion (Immuno-plate, Human IgG and Human IgM, Hyland Div., Travenol Laboratories, Inc., Costa Mesa, Calif.).

The medical records of 75 consecutive patients with vascular reactive sera were reviewed retrospectively. Since the population sample was biased at the outset (i.e. sera were submitted for ANA testing), the control sample was selected in the following manner. The laboratory accession number of each vascular reactive serum was determined and the serum with the next closest accession number which had been tested for ANA and which had been obtained from a patient in the same patient population was identified. The medical records from these additional 75 patients were then reviewed for control purposes.*

Latex fixation tests were done in serum dilutions of 1:40 through 1:81, 920 (12 tubes) using a previously described technique (Singer and Plotz, 1956).

* The patients were seen in the wards or clinics of Baylor College of Medicine, the Ben Taub General Hospital, Houston, Texas.

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Results

INCIDENCE AND RELATIONSHIP TO OTHER 'AUTOANTIBODIES'

The overall incidence of antivascular antibody (AVA) in the 5,295 sera tested was 6.6% (Table I). The ANA was present in 24.4% of the same sera. Not all specimens submitted for ANA were tested for RF. Therefore, analysis of 500 consecutive specimens submitted for ANA and RF was made (Table II). The overall incidence of antibody activities in this sample were RF 55%, ANA 25%, and AVA 4.2%. These data suggested that AVA positivity was not

dependent on either ANA or RF positivity. From Table I it would be predicted that 1.6% of sera positive for ANA would be positive for AVA if no association of ANA and AVA occurred; 0.5% were actually found. Similarly, from Table II it would be predicted that 2.3% of sera positive for RF would contain AVA and in actuality 2.6% were found.

CHARACTERIZATION OF AVA

Sera positive for AVA were positive on retesting. Further, several patients found to have a positive serum from whom serial samples were available were found to be positive over a period of months or years. For example, multiple sera obtained from one patient were positive over 6 years. Blocking tests with unconjugated antihuman globulin totally inhibited the reaction with five sera. Endpoint titrations were done on 12 sera and suggested that, in general, AVA was low titred, with a reciprocal dilution of 64 being the maximum titre observed in this series.

Fifty sera which reacted with mouse liver capillaries (Fig. 1a) were tested on mouse kidney sections, and again prominent staining of the vessels was observed including glomerular capillaries, peritubular capillaries, and arterioles (Fig. 1b). Mouse gastric mucosa generally did not stain, but in an occasional section of mouse stomach that contained small vessels cut in cross section, typical vascular staining was observed.

Separate mice were injected intravenously with a total of 0.5–0.6 ml of three positive sera in divided doses. 20 hours after the first injection and 2 hours after the final injection the mice were sacrificed and

Table I *Distribution of ANA and AVA in 5,295 sera*

Positive	No.	Per cent.	Predicted %
ANA	1,294	24.4	—
AVA	350	6.6	—
ANA + AVA	24	0.5	1.6

Table II *Distribution of RF, ANA, and AVA in 500 sera*

Positive	No.	Per cent.	Predicted %
RF	275	55.0	—
ANA	125	25.0	—
AVA	21	4.2	—
RF + AVA	13	2.6	2.3
ANA + AVA	5	1.0	1.1
RF + ANA + AVA	3	0.6	0.6

FIG. 1a *Antivasular antibody staining of mouse liver. $\times 415$*

FIG. 1b Antivasular antibody staining of mouse kidney. $\times 415$

kidney sections were reacted with antihuman Cohn Fraction II-FITC. No staining occurred. Five positive sera tested on sections of three different human kidneys produced negative results. Positive sera were separated into euglobulin and pseudoglobulin fractions and the vascular reactivity was found associated with the pseudoglobulin. That the reaction fixed complement was shown by positive staining with antihuman C3-FITC when fresh sera containing AVA were used or when sections were reacted sequentially with sera positive for AVA, fresh human sera, and anti-C3-FITC. Finally, sucrose density gradient centrifugation (Fig. 2) showed the association of AVA with those fractions containing IgG, and the absence of AVA in fractions containing IgM. These data established that AVA is immunoglobulin G and is capable of fixing C.

RELATIONSHIP OF AVA TO DISEASE

To assess the relationship of AVA to disease, the medical records of 75 consecutive patients with vascular reactive sera and 75 control patients were reviewed. Table III shows the wide spectrum of disease states in both groups. Connective tissue diseases predominated in both groups and rheumatoid arthritis (RA) was the single most frequently diagnosed disease. In the AVA positive group there were twenty patients with RA compared with eleven patients in the control group ($P < 0.05$). Within the connective tissue disease subgroups there were five patients with hepatitis B antigenaemia with AVA re-

FIG. 2 Sucrose density gradient centrifugation. Continuous sucrose gradients (10–40%, w/v) were layered with 0.5 ml serum samples and centrifuged for 18 hours as described in the text. The plotted values represent the means for all of the specimens. The arrows (fractions 3 and 4) indicate those fractions containing AVA.

active sera and none in the control group ($P < 0.05$). A highly negative correlation was observed with eight patients in the control group with a diagnosis of systemic lupus erythematosus (SLE) as compared with

Table III *Diagnostic categories of 150 patients*

Group	AVA+	Control
I Connective tissue diseases	44	50
II Vascular diseases	11	5
III Infectious diseases	5	4
IV Central nervous system diseases	5	3
V Renal diseases	3	4
VI Other (malignancies, dermatological, psychiatric, no diagnosis, etc.)	7	9
Totals	75	75

none in the AVA reactive group ($P < 0.005$). Another significant difference related to the presence or absence of ANA. There were 19 ANA containing sera in the control group as compared with three in the AVA reactive group ($P < 0.0005$).

The mean ages and ranges were comparable for the two groups, 41.8 years (range 1–73 years) in the AVA positive group and 40.4 years (range 2–87 years) in the control group. Both the sex and race ratios appeared to be partially reversed in the AVA positive group. The Negro to Caucasian ratio for the AVA group was 1.2 to 1.0 as compared with 3.7 to 1.0 in the controls. Likewise, in the AVA group female to male ratio was 1.5 to 1.0 as compared with the controls, 2.4 to 1.0.

Several unusual vascular diseases were observed in the AVA reactive group when compared to the controls. These included two cases of arterial thrombosis (carotid artery and central retinal artery), one case of granulomatous arteritis, one case of glomerulonephritis with hyperplastic arteritis, and two cases of severe, recurrent, and intractable thrombophlebitis (in one case, recurrent pulmonary embolization required the placement of an inferior vena caval umbrella). The number of patients studied was too small to determine whether there is a significant association between these varied vascular diseases and the presence of AVA.

The youngest patient with AVA was a 1-year-old

Caucasian male with congenital cytomegalic inclusion disease. He had been transfused repeatedly with plasma, so the origin of his AVA is unclear. The eldest patient with AVA was a 73-year-old Negro male with long-standing ankylosing spondylitis.

Since the majority of patients in the AVA reactive group had a connective tissue disease, we specifically reviewed available serological data from patients with established connective tissue diseases. Table IV contains the data from the rheumatic disease group. It is evident that patients with Reiter's syndrome, RA, and hepatitis B accounted for the largest number of positive sera. When compared to the other groups, P was < 0.005 .

CLINICAL SIGNIFICANCE

Since RA was one of the diseases most frequently associated with AVA, the clinical and laboratory data of a group of patients with RA that had been previously studied (Sharp, Lidsky, Collins, and Moreland, 1971) was reviewed and examined for correlations between AVA and the clinical, immunological, or roentgenological manifestations of their diseases. Depending on the parameter examined, between 58 and 75 patients were available for each analysis. The mean age for the patients with RA was 39.8 years, the female to male ratio was 1.57:1.00 (reflecting the fact that a large number of the patients in this study were from the Houston Veterans Administration Hospital). No positive or negative correlations were found in this study group between AVA and any of the following: time since onset, age, sex, race, nodules, joint space narrowing, joint destruction/time, total γ -globulin level, or presence of ANA. The mean RF titre was approximately one tube higher for those patients with AVA (8.33 *v.* 7.21). 24% of the patients with RA had AVA and 31% had ANA. The presence of both AVA and ANA was seen in 2.6% of the patients. These percentages are much higher than was found in the total serological survey (Table I). On the basis of chance, however, in this group one would predict both ANA and AVA to be positive in about 7%.

Table IV *Distribution of ANA and AVA in connective tissue diseases*

Diagnosis	No. of patients	ANA positive	AVA positive	Incidence AVA (%)
Reiter's syndrome	12	0	3	25
Rheumatoid arthritis	75	23	18	24
Hepatitis B associated with	20	2	4	20
(a) periarteritis nodosa	4	0	1	25
(b) polyarthritis	16	2	3	18.8
Degenerative joint disease	16	3	2	12.5
Gout	12	0	1	8.3
Systemic lupus erythematosus	25	24	1	4
Scleroderma	12	10	0	—
Periarteritis nodosa*	8	3	0	—
Dermatomyositis/polymyositis	8	3	0	—

* Exclusive of those with positive HB antigen.

Discussion

A number of autoantibodies (perhaps more accurately, heteroantibodies) have been described in the sera from patients with certain infectious, metabolic, neoplastic, and connective tissue diseases (Holborow, 1972; Strickland and Hooper, 1972; Tan and Pearson, 1972) in recent years. The characterization of the antigens involved and the mechanism of induction of these antibodies has proved to be most difficult. Speculation concerning the induction of these antibodies and their relation to the pathogenesis of human disease abounds. The finding of an antibody directed toward some antigenic site in or on murine capillaries was somewhat unexpected. The documented relationship of vasculitis to certain of the connective tissue diseases (Mongan, Cass, Jacox, and Vaughan, 1969; Norton and Nardo, 1970) suggested the need to pursue these observations in an attempt to define the reactivity and, if possible, relate it to the disease state. A recent report of rheumatic disease sera reactive with capillaries in the mouse kidney (Tan and Pearson, 1972) prompted this review of our experience with what we believe is the same phenomenon observed with capillaries in mouse liver. The fact that our experience with AVA extends over a number of years allowed for a retrospective review of the general patient population from whom reactive sera were obtained. As is the case with most autoantibodies, patients with various diseases were found to have reactive sera. The preponderance of rheumatoid sera which were reactive suggested the possibility of defining a subgroup on the basis of the presence or

absence of AVA. This, however, did not obtain. Of interest, but of unknown significance, was the number of patients with AVA positive sera who had involvement of different organ systems thought, on clinical grounds, to be of vascular origin. In this regard, the association of vasculitis and hepatitis B antigenaemia is of interest (Gocke, Hsu, Morgan, Bombardieri, Lockshin, and Christian, 1970). Whether murine and human vascular antigen(s) share common determinants is not known. The possibility exists and is perhaps worthy of study. It must be noted, however, that in the three different human kidneys we studied there was no evidence of vascular staining with AVA reactive sera. None of the kidneys were from patients with connective tissue diseases and it is possible that in some patients with connective tissue diseases hidden vascular antigens are exposed or neoantigens produced which cross-react with murine vessels, and which induce AVA.

Summary

A human serum protein which reacts with murine vessels is described. It has the characteristics of an antibody of the IgG class which fixes C and has been termed antivascular antibody. The overall incidence of AVA in more than 5,000 serum samples tested for antinuclear antibody was 6.6%. Its presence is associated with a variety of disease states, particularly connective tissue diseases, and most commonly with Reiter's syndrome, RA, and hepatitis B antigenaemia. It was not possible to relate AVA to a specific subpopulation of patients with RA.

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